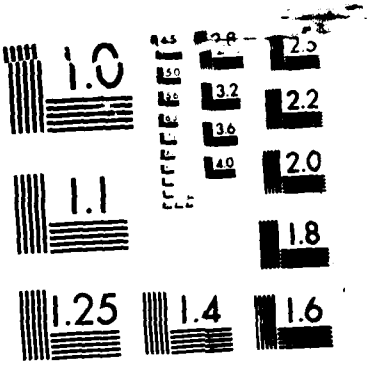
AD-A168 129 LIPOSOMAL-ENCAPSULATED STROMA-FREE HEMOGLOBIN AS A POTENTIAL BLOOD SUBSTITUTE(U) CALIFORNIA UNIV SAN FRANCISCO C A HUNT 31 MAR 82 DAMD17-79-C-9045 1/1

UNCLASSIFIED F/G 6/15 NL



AD-A168 129

DTIC ACCESSION NUMBER

PHOTOGRAPH THIS SHEET

LEVEL

INVENTORY

LIPOSOMAL-ENCAPSULATED STROMA-FREE HEMOGLOBIN as a POTENTIAL BLOOD SUBSTITUTE ANNUAL PROGRESS REPORT MARCH 31, 1982

DOCUMENT IDENTIFICATION

DISTRIBUTION STATEMENT A
Approved for public release
Distribution Unlimited

DISTRIBUTION STATEMENT

ACCESSION FOR		
NTIS	GRA&I	☑
DTIC	TAB	☐
UNANNOUNCED		☐
JUSTIFICATION		
BY		
DISTRIBUTION /		
AVAILABILITY CODES		
DIST	AVAIL AND/OR SPECIAL	
A-1		

DISTRIBUTION STAMP

DTIC ELECTE JUN 06 1986 S D

DATE ACCESSIONED

DATE RETURNED

86 6 5 087

DATE RECEIVED IN DTIC

REGISTERED OR CERTIFIED NO.

PHOTOGRAPH THIS SHEET AND RETURN TO DTIC-DDAC

DTIC FORM 70A DEC 83

DOCUMENT PROCESSING SHEET

PREVIOUS EDITION MAY BE USED UNTIL STOCK IS EXHAUSTED.

SUMMARY

Liposomal-Encapsulated Stroma-Free Hemoglobin as a Potential Blood Substitute

Annual Progress Report

by

C. Anthony Hunt, Ph.D.

March 31, 1982

(For the period April 1, 1981 through March 31, 1982

Supported by

U.S. Army Medical Research and Development Command
Acquisition Group
Fort Detrick, Frederick, MD. 21701

Contract No. DAMD17-79-C-9045
University of California
San Francisco, California 94143

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION / AVAILABILITY OF REPORT
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION University of California San Francisco 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) San Francisco, CA 94143	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING / SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-79-C-9045
8c. ADDRESS (City, State, and ZIP Code) Ft Detrick, Frederick, MD 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. / PROJECT NO. / TASK NO. / WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification)
(U) Liposomal-Encapsulated Stroma-Free Hemoglobin as a Potential Blood Substitute

12. PERSONAL AUTHOR(S)
C. Anthony Hunt, Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 4/1/81 TO 3/31/82	March 31, 1982	

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION UNCLASSIFIED
22a. NAME OF RESPONSIBLE INDIVIDUAL Mrs. Virginia Miller	22b. TELEPHONE (Include Area Code) 7325 / 22c. OFFICE SYMBOL SGRD-RMS

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

PROGRESS REPORT
DAMD17-17-C-9045

Liposomal-Encapsulated Stroma-Free Hemoglobin as a Potential Blood Substitute

C. Anthony Hunt. Ph.D.

Summary

Over the past year we have made considerable progress in optimizing preparation of NEOHEMOCYTES, NHC. All indications are that substantial further improvement is likely. At this time we can prepare NHC suspensions, suitable for infusion, that are equivalent in tissue delivery properties to either a 17.3g% SFH solution or to normal blood having a hematocrit 0.20. We know that the half-time for retention of NHC in the circulation increases dramatically as the amount transfused increases. Further, we know that circulation half-time is very is very sensitive to changes in the membrane composition and, to somewhat less extent, to their diameter.

One would prefer that circulation half-time be essentially the same for transfusions of either 10% or 90% of blood volume. To reach this goal we are preparing several "masking lipids." These are synthetic phospholipids with inert carbohydrate head groups; they are designed to minimize or prevent interaction or binding of NHC to tissues, and reduce the likelyhood of reticuloendotheial blockaid.

The prospects for NHC look very good! Potential antigenisity and undesirable effects on clotting time are potential problem areas that will require increasing attention.

Abbreviations

Hb: hemoglobin

SFH: Stroma free hemoglobin (supplied by LAIR)

Met-Hb: Met-Hemoglobin

DGP: 2,3-diphosphoglycerate

IHP: inonsitolhexaphosphate

HCL: hemoglobin containing liposomes: NEOHEMOCYTES

n: the Hill number for Hb/O_2 binding

NHC: Neohemocyte(s)

mosm: miliosmolar

PC: phosphatidylcholine

PA: phosphatidic acid

CH: cholesterol

T/ (αT): α-tocopherol

w/DPG: with DPG

w/o DPG: without DPG

OTS: oxygen transport system

Hct: hematocrit

Types of NHC discussed in This Report:

NHC:C-1(x). These NHC have a composition of PC/PA/CH/T in a molar ratio of 4:1:5:0.1 with an average diameter of about 0.4 μm. The concentration of SFH in the original solution to be encapsulated was Xg%. The osmolarity of the starting SFH solution was 30mosm resulting from SFH and pH7.5 phosphate buffer. After preparation these NHC were slowly dialyzed against a 300 mosm dextrose to equilibrate internal and external osmolarity.

NHC:C-2(x). The composition an size of these NHC are the same as the NHC:C-1(x). The concentration of SFH in the starting solution is Xg%; however, the osmolarity of this starting solution is 300 mosm. Phosphate contributes 30 mosm, the remainder is due to dextrose. The internal aqueous phase of these NHC (the starting solution of SFH) is iso-osmotic with plasma.

NHC:C-3(x). The composition, size and SFH concentration of these NHC are the same as the NHC:C-1(x). However, the osmolarity of this starting solution is 300 mosm composed only of the SFH and the phosphate buffer (much higher ionic strength than NHC:C-2(x)); the initial aqueous phase of these NHC (the starting solution of SFH) is iso-osmotic with plasma.

Technical Objectives

Activity TO DATE:

Our objectives this year have been:

1. Improve the amount of SFH encapsulated in NHC.
    a. Investigate various techniques to adjust the osmolarity of NHC to that of plasma when hypo-osmotic solutions are used to prepare NHC.
    b. Further evaluate procedures to separate Hb-rich from Hb-poor NHC.
2. Increase the P_{50} of entrapped SFH by co-encapsulating either DPG or IHP.
3. Establishing the viscosity of NHC.
4. Establishing the performance of NHC in standard *in vitro* clotting tests.
5. Develop techniques to further minimize interaction of NHC with tissues *in vivo*.
6. Determine the degree to which the encapsulated SFH modifies the organ disposition pattern of liposomes having a fixed composition and size.

Results

I. Improved SFH Encapsulation

In our last report we indicated that the amount of SFH encapsulated per mol of total lipid was a function of several variables (Scheme I). To maximize SFH encapsulation for a fixed NHC size one must: (a) keep the concentration of met-Hb below 5%, (b) use a higher initial concentration of SFH (we have carried out encapsulations using as high as 30g% SFH), (c) keep the total osmolarity of the SFH solution as low as possible, (d) minimize ionic strength, (e) lower the amount of lipid used per unit volume of SFH (although) this improves encapsulation as defined above it decreases the efficiency of encapsulation), (f) match the densities of the lipid-solvent and aqueous-SFH phases, (g) minimize the size of emulsion particles, e.g., emulsify for a longer time, (h) minimize formation of met-Hb, e.g. emulsify for a shorter time.

Unfortunately one can not optimize each variable independent of the others because they are interdependent (consider g and h). For example the quality of emulsion properties decrease as the total osmolarity of the aqueous phase and/or the ionic strength component of osmolarity increases, yet the final product must be iso-osmotic with plasma and should have a relatively high buffer capacity.

At this stage the most important question seems to be whether to prepare hypoosmotic NHC (e.g. NHC:C-1(x)) and adjust internal osmolarity in a subsequent step or to simply prepare iso-osmotic NHC. We currently have no preferred direction, but are actively investigating each.

It now seems very likely that the final OTS preparation procedure will involve separation of Hb-rich from Hb-poor NHC. We now do this in two ways: (i) adjust the density of the final suspending buffer so that only HCL with densities greater than some value are centrifuged down (all others are centrifuged up), or (ii) centrifuged at five g's for a fixed time. Each procedure gives somewhat different Hb-rich NHC, but not substantially different.

The importance of separation of Hb-rich from Hb-poor (Scheme I) can not be overstated. Consider a typical packed NHC suspension with a hematocrit of 98. If we isolate the denser half of these NHC we will find that they contain about 66 to 77% of the total encapsulated SFH and only 20 to 35% of the total lipid present. Clearly, the Hb-rich NHC are much closer to the ideal OTS. Results of several studies are summarized in Table I.

The ability of some NHC suspensions described in Table I to deliver oxygen to tissues in vivo has been estimated and compared to similar estimates for simple solutions of SFH and normal blood in Table II. Clearly, these NHC have the potential to function as a superior resuscitative fluid.

II. Co-Encapsulation of DPG or IHP.

As can be seen in Table I we now encapsulate either DPG or IHP in almost all current NHC to improve both n and P_{50}. However, there is a price for this improvement, as indicated in Table III. For a fixed amount of lipid for encapsulating a fixed volume of starting SFH solution (constant SFH concentration) as the molar ratio of DPG (or IHP) to SFH increases the final fraction of the SFH solution that is encapsulated decreases. The gain in increased n and P_{50} relative to decreased encapsulation seems to peak for a DPG/SFH ratio of about 1.6 to 1. Studies need to be undertaken to obtain the improved n and P_{50} values without reduction in encapsulation.

III. Neohemocyte Viscosity

Table I. Properties of Various NHC[1]

Type of NHC	P50	n	Vol $\%O_2$[2]	g %Hb[2]	Starting SFH Concentration	% SFH[3] Encapsulated
Control (8.3% SFH)	12.6	2.05	11.1	8.3	8.3	-
NHC:C-1(8.3)[4] w/DPG	22.1	1.75	4.87	3.63	8.3	33.9
NHC:C-1(8.3)[5] (Hb-rich)w/DPG	22.1	1.75	6.96	5.19	8.3	23.7
NHC:C-1(8.3)[4] w/IHP	25.7	1.04	4.45	3.32	8.3	16
NHC:C-1(8.3)[5] (Hb-rich)w/IHP	25.7	1.04	6.36	4.74	8.3	11.2
NHC:C-1(33)[4] w/DPG	20.6	1.76	11.52	8.60	33	3.9
NHC:C-1(33)[5] (Hb-rich)w/DPG	20.6	1.76	16.46	12.3	33	2.7
NHC:C-2(8.3)[4] w/DPG	22.9	1.75	8.21	6.13	8.3	33.2
NHC:C-2(8.3)[5] (Hb-rich)w/DPG	22.9	1.75	11.7	8.76	8.3	23.2
NHC:C-3(8.3)[4] w/DPG	21.2	1.79	2.81	2.09	8.3	11.3
NHC:C-3(8.3)[5] (Hb-rich)w/DPG	21.2	1.79	4.01	2.99	8.3	7.9

[1]All P50 and $\%O_2$ values were normalized to central SFH values to remove differences between days and yeast solutions. All NHC were composed of PC/PA/CH/T in the ratio 4:1:5:0:1. pH was held constant at 7.5. The molar ratio of DPG or IHP to SFH in the starting SFH solutions were fixed at 1.6 to 1. Met-Hb levels in control SFH solutions averages 2.5%; for each of the NHC listed Met-Hb averaged 5% in the final, tested suspension. In all cases 50 μmoles of lipid (total) were used per ml of the starting SFH solution.

[2]These values are for an aliquot of NHC having a hematocrit of 0.95 to 1.00.

[3]These values represent the percent of the original SFH entrapped in the final, tested NHC suspension.

[4]This suspension contains both Hb-rich and Hb-poor NHC.

[5]These are the Hb-rich NHC; these values are based on average determinations where Hb-rich NHC consist of ½ the volume of the original NHC; they are that half with the higher density. On average the more dense half contains 70% of the encapsulated SFH (the basis of these numbers).

Table II. A Comparison (Calculated) of the Ability Neohemocytes Suspensions, SFH Solutions and Whole Blood to Deliver Oxygen to Tissues.

For these calculations we assume that arterial pO_2 = 100 mm Hg and venous pO_2 = 40 mm Hg, that each suspension's oxygen binding properties follow the simple Hill equation, that P_{50} = 22.85 and n = 1.75 for NHC, that P_{50} = 12.64 and n = 2.05 for SFH, and that P_{50} = 26.0 and n = 2.8 for blood. What we have done is to calculate the amount of O_2 that the NHC suspensions might deliver to tissues (average human) in one cycle through the body and then determine the percent of SFH or the hematocrit of blood necessary to deliver the same amount of oxygen.

NHC Suspensions	O_2 (ml) Released Per Pass	Equivalent SFH Conc.	Equivalent Blood Hct
Normal NHC			
Hct = 50	0.84	8.7 g%	10
Hct = 70	1.17	12.1 g%	14
Hb-rich NHC			
Hct = 50	1.20	12.4 g%	14.3
Hct = 70	1.67	17.3 g%	20.0

We've tested the viscosity of several NHC suspensions. The bottom line is that NHC suspensions with Hct's equivalent to that of a blood sample have viscosities considerable less than blood. For those NHC listed in Table I, suspensions having apparent hematocrits of approximately 0.70 have viscosities essentially equivalent to that of whole blood. More work is needed, especially as NHC composition is changed.

IV. Effects of Neohemocytes on Coagulation Times

Clearly the best strategy for evaluating the effects of NHC on coagulation time is to carry out studies in vivo. Although planned, this has not been done. We have, however, carried out preliminary studies in vitro (Table III). The results indicate that NHC have a modest anti-coagulant effect, which in general should not be a problem.

Table III. Performance Times* of Various Coagulation Tests.

Sample	PT	aPTT	PTT	hCT
Saline control	11.6 ± 0.26	26.4 ± 1.11	64.0 ± 8.5	34.3 ± 0.94
SFH	12.7 ± 0.22	24.4 ± 2.16	98.6 ± 12.26	45.2 ± 2.22
Liposomes	14.9 ± 0.06	35.1 ± 0.98	82.8 ± 1.99	45.6 ± 2.59
Neohemocytes	15.6 ± 0.32	30.5 ± 0.64	108.1 ± 7.89	53.0 ± 2.28

*All times in seconds

V. Reduction of Neohemocytes with Tissues In Vivo

We are now taking a new approach to this goal. Several early in vivo studies indicated that attachment of inert carbohydrates to liposome surfaces improves their plasma retention times. Results from other laboratories suggest that liposomes prepared with sphingomyelin (SH) in place of

some or all of the PC also gives liposomes with increased plasma retention times. We are following both leads. A refinement of the carbohydrate approach is to prepare a lipid with an inert carbohydrate head-group, a "masking lipid," ML. An example would be phosphatidylethanolamine with a low molecular weight dextran or inulin attached to the phospholipid amine. The basic idea is that such carbohydrates, because of their structure and possibly their non-reducing nature, will retain their inert properties when suitable attached to a lipid component of an NHC and thereby reduce the non-specific interaction of the NHC with tissues which would result in a prolonged circulation half-time. Synthesis of such MLs is difficult, but never the less is is progress.

VI. Effects of Encapsulated SFH on Liposome Disposition.

A critical assumption in our overall research strategy has been that the disposition properties of the final preferred NHC would be the same irrespective of the concentration of the encapsulated SFH, including the case for non encapsulated SFH. This is important for two reasons: (a) one more potential variable is eliminated in future studies, (b) in vivo disposition results can be obtained faster because the preparation time can be considerably shortened. We have tested this assumption and found it to be reasonably valid. Thus, we can state the following about the disposition properties of NHC at tracer doses (equivalent to bolus doses of 1 to 50 ml of a suspension with a Hct=50 in a 70 kg man).

(a) As the administered total surface area of the particles increases, first vascular, then hepatic, then spleeninc uptake/binding becomes saturated resulting in a large fraction of the dose remaining in the circulation for longer periods of time (relative to long-term goals a positive result).

(b) The organ and total body disposition properties were essentially the same for liposomes having mean diameters between 0.1 and 0.5 μm (a positive result).

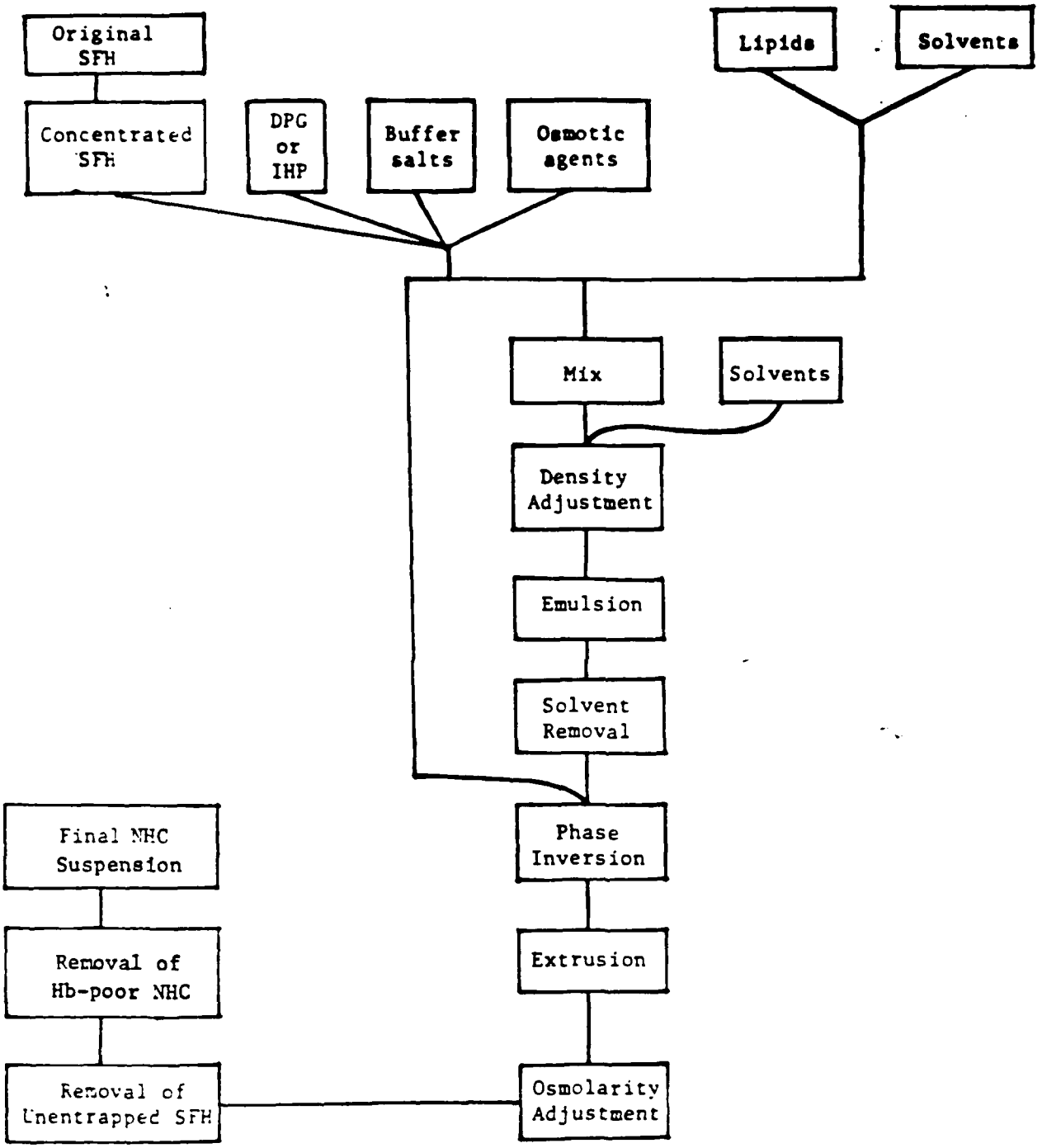
(c) Liposomes of very similar diameter interact with the same tissue binding/uptake sites, however, as liposome diameters become significantly different so do the binding/uptake sites. Thus the dose of a heterogenous liposome (or NHC) suspension needed to saturate tissue binding/uptake would be dramatically larger than the dose of a more homogenous suspension (a somewhat negative result).

(d) For the NHC composition used in these studies RES uptake is neither rapid nor extensive (a positive result).

Future Objectives

1. Evaluate the in vivo function of NHC following incomplete and complete transfusions in rats.
2. Determine the ability of several "masking lipids" to reduce tissue interaction of NHC in vivo and increase their circulation half-time.
3. Improve the physicochemical properties of Neohemocytes by increasing the encapsulated Hb fraction and optimizing lipid composition.
4. Scale-up preparation to 100 ml batches.
5. Evaluate shelf-life and stability.
6. Carry out acute toxicity study.
7. Establish the rate of met-Hb formation in vivo for remaining NHC.
8. Assess effect of NHC on the reticuloendothelial system.

Scheme I: Flow Chart for Preparation of NEOHEMOCYTES



END

DTIC

7-86